# LOCALISATION OF BIOACTIVE METABOLITES IN MARINE SPONGES

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Marine natural produet ehemists have often proposed that bioaetive sponge metabolites are produeed by symbiotie micro-organisms. This paper diseusses the rationale for these proposals, reviews the strengths and weaknesses of methods that are available to test such hypotheses and reports some experimental studies. The eonelusion reached from the researeh to date is that it is too early to make generalisations eoneerning either the role of symbionts in the biosynthesis of sponge metabolites or even the best techniques for studying the eellular localisation of bioaetive metabolites. ❒ *Porifera, bioactive metabolites, cyanobacteria, filamentous eubacteria, symbiosis, Aplysina fistularis, Dysidea herbacea, Theonella swinhoei, Oceanapia sagittaria, Jaspis splendens.*`

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Sponges are exceptionally good synthetic chemists. They can make chemicals of extreme toxicity and/or deterrent value that have undoubtedly contributed to their survival over the ages. But they may not always have acted alone. We now know that some sponges harbor populations of symbiotic micro-organisms that produce the chemicals thought to defend the sponge from competition or predation. However, it is clear that this situation is less common than the marine natural products literature would have us believe. This paper reviews the methods used to determine the cellular location of natural products in sponges and presents some recent results from our laboratory that either confirm or deny the production of 'sponge metabolites' by symbiotic microbes.

SYMBIOSIS AS SEEN FROM THE VIEWPOINT OF CHEMISTRY. The history of natural products chemistry has been driven by the use of natural products to treat diseases. First came an interest in plant products such as digitalis and morphine, but this was superseded in the second half of this century by the discovery of a plethora of immensely important antibiotics and other drugs obtained by the fermentation of microbes. Chemists became indoctrinated with the concept that micro-organisms could provide the needs of the pharmaceutical industry, which for a long period of time was not far from the truth. Then came the discovery that marine organisms could provide many new classes of natural products that incorporated new and unexpected structural motifs. Within this group, sponges have provided not only the best source of novel compounds but also the greatest occurrence of potential pharmaceuticals (Faulkner, 1998, and references therein). However, when chemists compared the structures of sponge metabolites with those of compounds in the literature, they found many structures that were very similar to those of microbial metabolites. When chemists saw scanning electron micrographs of sponges that contained large numbers of micro-organisms, they felt justified in proposing that compounds resembling microbial metabolites were in fact of microbial origin. Furthermore, when closely related or identical compounds were found in different phyla and there was no evidence of transmission of the chemicals through the food chain, they proposed that these compounds might be produced by the same or similar micro-organisms endemic to hosts of different phyla. These hypotheses set the stage for a careful investigation of the role of symbiotic microbes in the production of 'sponge metabolites'.

LOCALIZATION OF SPONGE METABOLITES. There are two basic strategies for determining the location of specific metabolites in sponges: detection of compounds using microscopy, or cell separation followed by chemical analysis of each cell fraction. The strategy selected generally depends on the molecular properties of the compound to be investigated. Compounds that contain heavy elements such as bromine or iodine

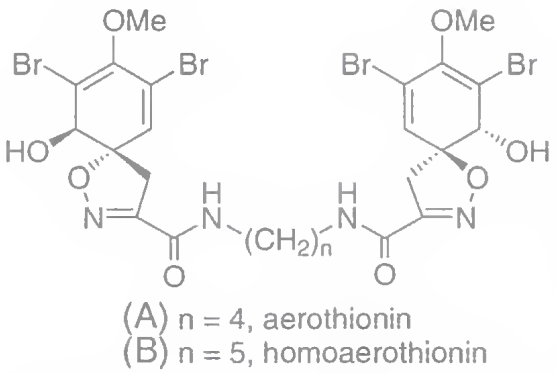



FIG. 1. Tetrabrominated metabolites. A, aerothionin. B, homoaerothionin.

can be detected by using an energy dispersive spectroscopy (EDS) detector on a scanning electron microscope (SEM) or a scanning transmission electron microscope (STEM). In theory, one could use the same technique to determine the location of compounds containing chlorine or sulfur but, in practice, the levels of chloride and sulfate ions in seawater preclude its use with marine specimens. Fluorescent compounds can be conveniently detected by fluorescence microscopy and by laser scanning confocal microscopy, but this technique is susceptable to problems caused by background fluorescence due to photosynthetic pigments and general autofluorescence of cells. The method of immunostaining using polyclonal antibodies to bind to a specific compound is common in cellular biology but prior to a report at this symposium (Ilan, 1998) and one other recent paper (Perry et al., 1998) had not been applied to study sponge metabolites. Finally, there is the possibility that specific compounds may be detected in cell preparations using secondary ion mass spectrometry in conjunction with tandem mass spectrometry. The latter two methods, both of which can be fine-tuned to detect individual compounds, could offer considerable advantages over methods that rely on detecting a class of compounds.

Cell separation methods take advantage of our ability to analyse the chemical content of fixed cells but suffer from the disadvantage that fractions containing only a single cell type may be difficult to prepare. Sponge tissues can be dissociated by enzymatic digestion or mechanical disruption in calcium-magnesium free seawater using squeezing, sieving, simple mincing, vigorous stirring, or even a juicer. The dissociated cells can then be fixed, which stabilises the cells during the period between collection and analysis. It is a relatively simple matter to separate cyanobacteria using a cell sorter to distinguish fluoresecent from non-fluorescent cells but this method does not distinguish between sponge and eubacterial cells. Cell types can also be separated by density using either differential centrifugation or Ficoll or Percoll density gradients. It has been our experience that repeated centrifugation is required to produce fractions of reasonable purity and that the different sponge cell types are difficult to separate on the basis of density. Nonetheless, filamentous bacteria, mixed sponge cells and mixed unicellular bacterial cells can all be enriched to ca. 90% purity using centrifugation. To detect the compounds of interest, each cell fraction is then extracted individually and analyzed using two or more of the following techniques: mass spectrometry (MS), which can be combined with high performance liquid chromatography (HPLC) or gas chromatography (GC), HPLC using a diode array detector to measure the UV spectrum, and $^{1}$H NMR spectrometry.

## RESULTS AND DISCUSSION

The tetrabrominated metabolites aerothionin (Fig. 1A) and homoaerothionin (Fig. 1B), which occur as a 10:1 mixture in a shallow-water specimen of *Aplysina fistularis* from La Jolla, were ideal candidates for study using energy dispersive spectroscopy because the molecules contain such a high concentration of bromine. Analysis of the STEM images using energy dispersive X-ray analysis revealed a 20-fold larger concentration of bromine in spherulous cells than in bacterial or other sponge cells, which were both at background levels. We therefore argued that the brominated metabolites (Fig. 1A-B) were produced and stored in spherulous cells (Thompson et al., 1983).

There are two major chemotypes of *Dysidea herbacea*; one contains both sesquiterpenes and metabolites biosynthesised from polychlorinated amino acids, the other produces only polybrominated biphenyl ethers and lacks terpenes. Very significant populations of cyanobacteria are found in both chemotypes and in both cases, the cyanobacterium is considered to be *Oscillatoria spongelliae*. The fluorescent cyanobacterial cells were separated from all other non-fluorescent cells using a cell sorter and

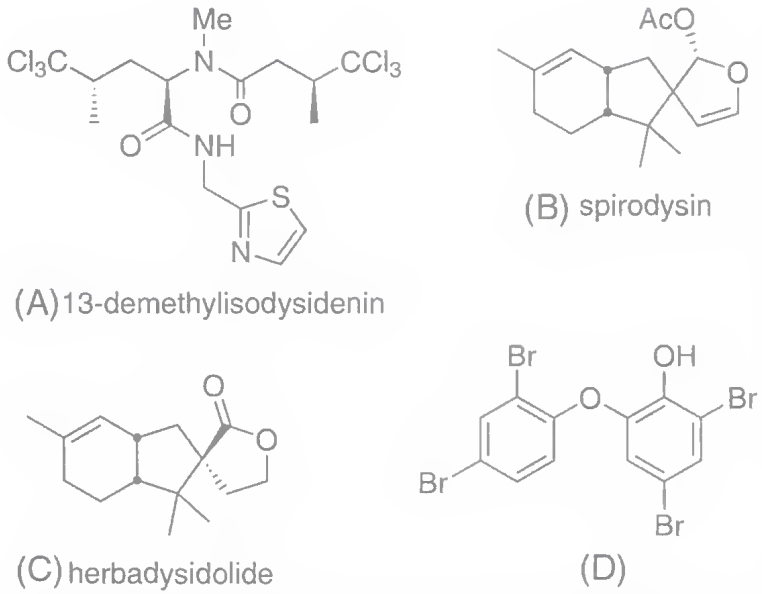



FIG. 2. Metabolites from two major chemotypes of *Dysidea herbacea*. A, 13-demethylisodysidenin. B, spirodysin. C, herbadysidolide. D, polybrominated biphenyl ether.

the chemical content of each cell type was analysed by $^{1}$H NMR spectroscopy and GC-MS. In a specimen of *D. herbacea* from Heron Island, 13-demethylisodysidenin (Fig. 2A), a polychlorinated amino acid derivative, was extracted from the cyanobacterial cell fraction while the sesquiterpenes spirodysin (Fig. 2B) and herbadysidolide (Fig. 2C) were detected in the fraction that contained sponge and bacterial cells (Unson & Faulkner, 1993). Garson and coworkers recently separated the sponge cells using a Percoll density gradient and showed that the sesquiterpenes were located in a fraction containing archaeocytes and choanocytes (Flowers et al., 1998). A similar analysis of a specimen of *D. herbacea* from Palau revealed that the polybrominated biphenyl ether (Fig. 2D) was located not only in the cyanobacterial fraction but also as crystals situated just below the surface of the sponge (Unson et al., 1994).

The sponge cells were considered to be devoid of brominated metabolites, although it was possible to detect a very low level of the polybrominated biphenyl ether (Fig. 2D), which was consistent with the presence of a small number of cyanobacterial cells that remained as contaminants in the sponge cell fraction. Having shown that cyanobacteria are responsible for the halogenated chemicals in the two chemotypes of *D. herbacea*, there is now a need to analyse the 16S rRNA sequences of representative samples to determine whether the cyanobacteria represent two different strains of *O. spongelliae* or different cyanobacterial species, which are two of several possible explanations of the chemical diversity. The diversity of chemistry assigned to *Dysidea* spp. may also provide a good rationale for a sponge taxonomist to re-examine the genus and particularly the chemists' voucher specimens.

The lithistid sponge *Theonella swinhoei* has provided chemists with an almost unequalled source of highly bioactive chemicals (Bewley & Faulkner, 1998). Our interest in this sponge was piqued by the structural similarity between swinholide A (Fig. 3A), which had previously been isolated from *T. swinhoei*, and the cyanobacterial product scytophycin C (Fig. 3B) and by the fact that the cyclic peptides of *T. swinhoei*, such as theopalauamide (Fig. 3C)(Schmidt et al., 1998), contain aromatic ß-amino acids similar to those found in some cyanobacterial cyclic peptides (Ishibashi et al., 1986; Kitagawa et al., 1990; Bewley & Faulkner, 1998).

This led to a suggestion that the prominent filamentous micro-organisms in *T. swinhoei* were cyanobacteria that produced both groups of compounds (e.g. Kobayashi & Ishibashi, 1993; Fusetani & Matsunaga, 1993). We had reason to suspect that this assumption was incorrect because the sponges were often found in caves, the filaments were found in the interior of the sponge, away from the light, and extracts of the endosomal tissue of the sponge did not appear to contain sufficient chlorophyll pigments. In a specimen of *T. swinhoei* from Palau, there were unicellular cyanobacteria (*Aphanocapsa feldmanni*) in the ectosome, which was peeled away and examined separately. The ectosomal tissues were dissociated and the cyanobacteria were separated using differential centrifugation, but they did not contain the metabolites of interest. The endosomal tissues were dissociated, fixed in a mixture of formalin and glutaraldehyde in artificial seawater, and separated using

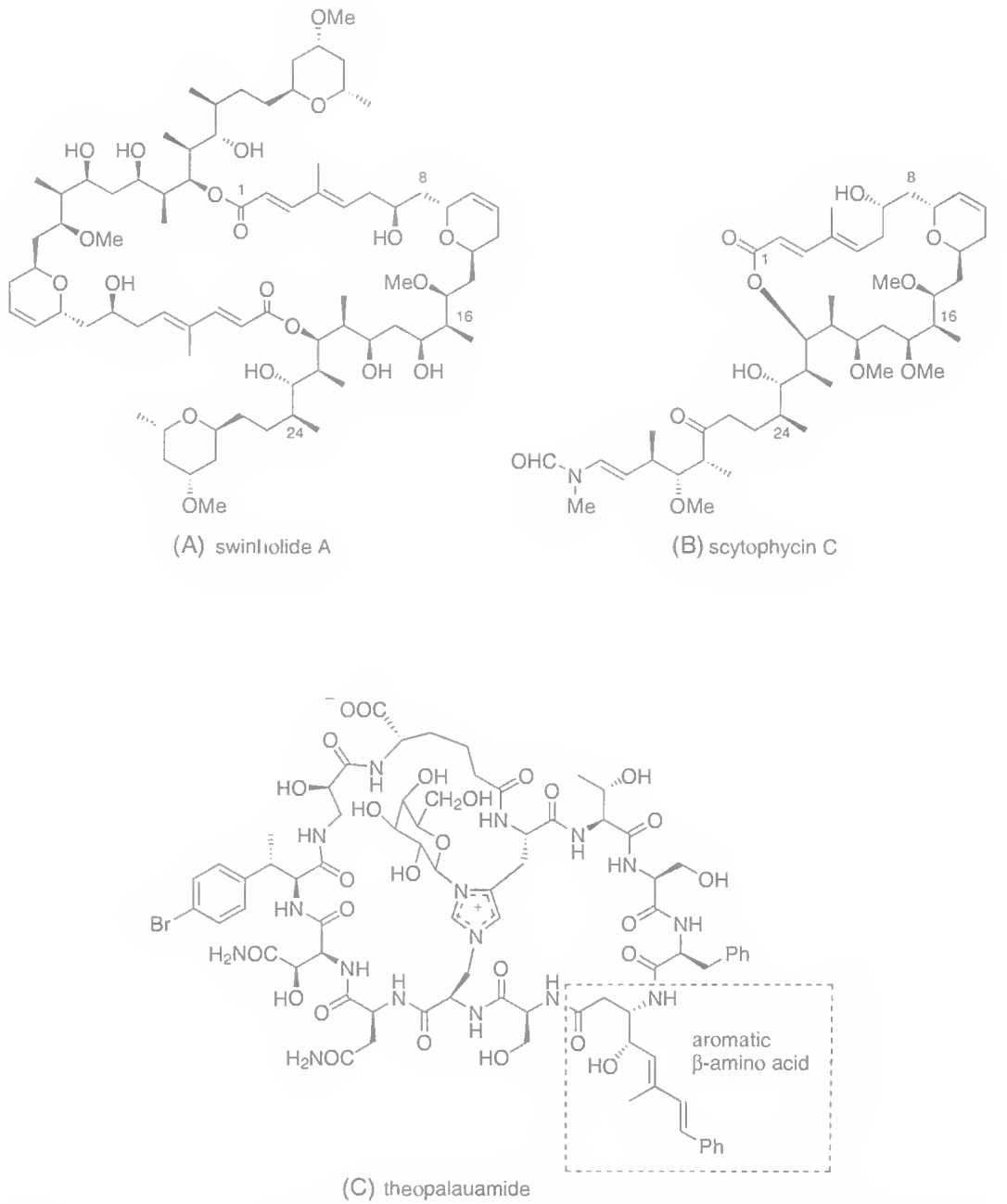



FIG. 3. Metabolites from the lithistid sponge *Theonella swinhoei*. A, swinholide A, which partially resembles scytophycin C. B, cyanobacterial product scytophycin C. C, theopalauamide.

differential centrifugation into three fractions containing mixed sponge cells, a filamentous bacterium, and mixed unicellular bacteria. The cell fractions were thoroughly washed, then extracted to obtain crude extracts that were analysed by $^{1}$H NMR and HPLC. Theopalauamide (Fig. 3C) was found to be present in about 4% dry weight in the filaments, which were examined by TEM and found not to be cyanobacteria, since they lacked the thylakoid

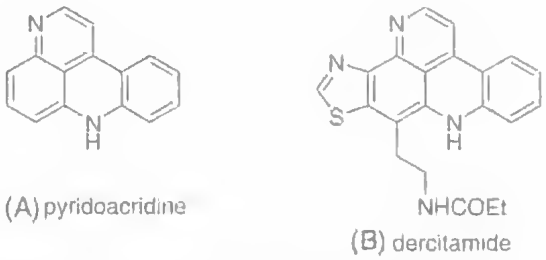
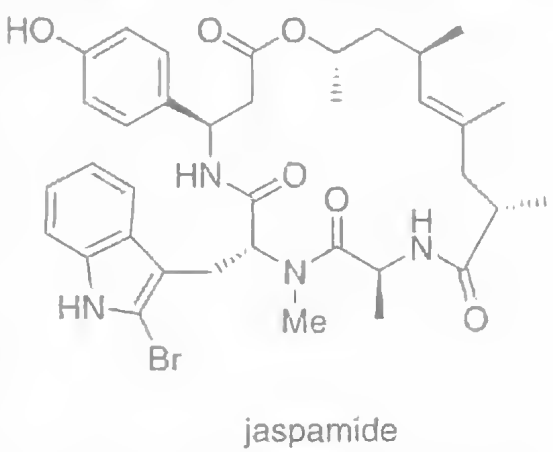



FIG. 4. A, pyridoacridine skeleton. B, dercitamide.

structures that house the photosynthetic apparatus of cyanobacteria (Bewley et al., 1996). We are currently characterising the cubacterial filaments using 16S rRNA analysis. Swinholide A (Fig. 3A) was extracted from the unicellular bacterial fraction, which contained many morphologically distinct bacteria. A recent re-examination of the $^{1}$H NMR spectrum of the unicellular bacterial fraction revealed the presence of the 4-methylene sterols that are typical of *Theonella* spp., but we need to reconfirm that result because sterols are not usually produced by cultured bacteria. Both the sponge cells and the cyanobacterium *Aphanocapsa feldmanni* appeared to be devoid of bioactive metabolites.

The pyridoacridine alkaloids, which all possess the same underlying tetracyclic aromatic ring system (Fig. 4A), are examples of a class of metabolites that have been found in four different marine phyla, but predominantly in sponges and ascidians (Molinski, 1993). They have frequently been proposed to be metabolites of undesignated microbial populations that might occur as symbionts in the different phyla. We felt that there might be an alternative explanation based on the evolution of similar biosynthetic schemes in different phyla, in part because polyaromatic compounds are the most stable products that can arise from their presumed mode of biosynthesis (Steffan et al., 1993). Dercitamide (Fig. 4B) has been reported from both sponges and ascidians (Gunawardana et al., 1992; Carroll & Scheuer, 1990) — the latter authors referring to dercitamide as kuanoniamine C - and we have isolated it as the major metabolite of the sponge *Oceanapia sagittaria* (Salomon & Faulkner, 1996). Dercitamide is an interesting pigment that changes color from yellow in neutral or basic solution (pH > 7) to red in acidic solution (pH < 6) and has a fluorescence spectrum that is also pH dependent. Using a light microscope, one can observe a change in the color of the sponge tissue when a section is acidified using trifluoroacetic acid vapor. A similar pH dependency was noted when sections were observed using fluorescence microscopy, but there was so much fluorescence from cells that were out-of-plane that it was impossible to clearly image the cells containing dercitamide. Examination of both thick sections and enriched cell fractions using a confocal microscope under both neutral and acidic pH conditions led to the conclusion that dercitamide was localised in sponge cells containing between ten and twenty spherical inclusions. Transmission electron microscopy was employed to show that there were no intracellular bacteria that could be responsible for the chemistry (Fig.6). The dercitamide-containing cells were characterised by TEM analysis, although they have not been classified as a particular type of sponge cell. This appears to be the first time that confocal microscopy has been employed to locate marine natural products on the basis of their autofluorescence. Research is in progress to determine the cellular location of pyridoacridine alkaloids in ascidians.

Not every study has resulted in an unambiguous localisation of metabolites. The cyclic depsipeptide jaspamide (Fig. 5) is a cytotoxic metabolite of *Jaspis splendens* (De Laubenfels, 1954), referred to as *Jaspis* sp. in our earlier chemistry paper (Zabriskie et al., 1986), that has been proposed both as a chemotaxonomic marker and to be of microbial origin. It is interesting to note that jaspamide (Fig. 5) was also isolated from a completely different sponge, *Auletta* cf. *constricta* (Crews et al., 1994). The sponge was dissociated in a juicer, a technique previously used successfully on *T. swinhoei*, followed by




jaspamide

FIG. 5. Cyclic depsipeptide jaspamide from *Jaspis splendens*.

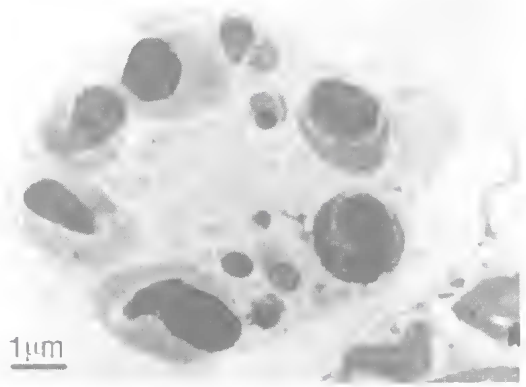



FIG. 6. Micrograph of a dercitamide-containing inclusional sponge cell from *Oceanapia sagittaria* that shows the absence of intracellular symbionts.

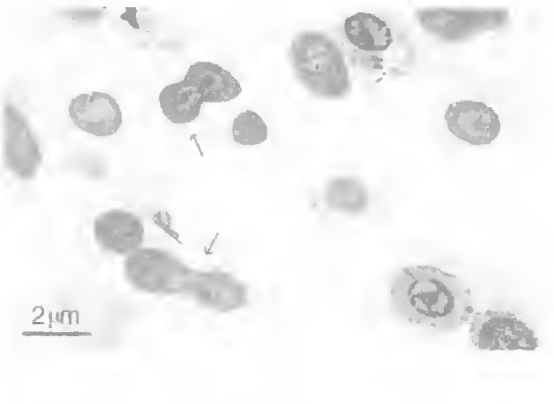



FIG. 7. Micrograph of dissociated cells of *Jaspis splendens*. Arrows indicate the unidentified symbionts that do not contain jaspamide.

fixation and cell separation using differential centrifugation. Jaspamide was not detected in extracts of an unidentified extracellular 'symbiont' (Fig. 7) or associated micro-organisms, which represented a large proportion of the whole sponge biomass, but was isolated in nearly 4% yield from a fraction containing small (ca. 500nm) orange bodies and cellular debris. The identity of the orange bodies is uncertain, but we have evidence that the dissociation process may have ruptured the sponge cells with concomitant release of the orange bodies. Examination of newly acquired sponge material by light microscopy revealed the presence of numerous small orange inclusions within the sponge cells. We now believe that jaspamide (Fig. 5) is located within sponge cells and further research is in progress to test this hypothesis.

## CONCLUSIONS

The major conclusion that we have reached during our studies of the role of symbionts in the production of sponge metabolites is that it is extremely dangerous to make any general statements about the sources of bioactive metabolites. In essence, each compound of interest requires an individual study to determine its source. The results that we and others have generated indicate that it is possible to detect specific compounds or classes of compounds in either symbiont or sponge cell fractions and that the concentrations of secondary metabolites in isolated cell fractions can be spectacularly high. However, it is often difficult to determine which sponge cell type produces the metabolite and it is nearly impossible to define a particular unicellular bacterium as the source of a bioactive compound. The latter will undoubtedly require the culture of symbiotic micro-organisms, which is the goal of several research groups. In order to accomplish this goal, we have proposed a strategy that involves identification of the symbionts from their 16S rRNA sequence (Brantley et al., 1995) before attempting to culture them using media that are suitable for culturing their nearest relatives. While the ultimate goal is to culture symbionts that produce pharmacologically-active sponge metabolites in order to speed their pharmaceutical development, information gained from cellular localisation studies can also be useful in chemotaxonomy and the understanding of bio-synthetic pathways that may have influenced the evolution of symbioses within sponges.

## ACKNOWLEDGEMENTS

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